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Prevalence of *CDKN2A* gene deletion in Breast Cancer: An Indian experience

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Abstract

Background:
Breast cancer is the most common cancer in females, found to be associated with mutations in various genes. *CDKN2A* is one such tumor suppressor gene which is altered in a wide range of human neoplasms and most frequently shown to play a role in breast cancer. It may be used in the development of a targeted therapy that could result in improved patient prognostication and outcome. In the present study the frequency of *CDKN2A* gene deletion and its relationship with clinicopathological features were examined in breast cancer patients.

Method: Histopathologically confirmed breast cancer patients were included for *CDKN2A* gene deletion study by Fluorescence i*n situ* hybridization (FISH) method. Formalin fixed paraffin embedded tissues were used for the assays. A total of 30 breast cancer patients were included. FISH was performed using P16 (CDKN2A) deletion probe kit. Images were captured by Epi-fluorescence microscope.

Result: In breast cancer patients, *CDKN2A* gene was deleted in 22 patients (73%) with 17 hemizygous and 5 homozygous deletions, while 8 patients (27%) had normal intact form. *CDKN2A* gene deletion in breast cancer was found to be significantly inversely associated with tumor grade, presence of perineural invasion, perinodal extension and vascular permeation.

Summary and Conclusion: *CDKN2A* gene deletion was associated with absence of known worse prognosticators indicating that the gene expression and not loss was linked to adverse tumor parameters. Hence, detection of *CDKN2A* gene deletion could help to stratify the specific subsets of patients into high risk and low risk groups. *CDKN2A* gene deletion can also help as the pre-screening tool prior to gene evaluation tests.

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INTRODUCTION

Breast cancer has thrived to become the most common cancer representing 1 in 4 cancers diagnosed among women worldwide, according to Globocan 2020. Despite advances in oncology, breast cancer remains the leading cause of cancer deaths in female globally (Sung et al, 2021). It is, therefore, a prerequisite to define and understand events resulting in breast carcinogenesis at the molecular level. It has been detected that various chromosome are affected by a higher frequency of structural or numerical abnormalities in breast cancer. (Brenner and Aldaz, 1995). At the molecular level, several somatic mutations have also been described affecting various oncogenes and tumor suppressor genes. Such mutations in cell cycle-related genes such as *TP53, CDKN2A, RB1* and *BRCA1* usually lead to tumor formation.

CDKN2A gene localized at chromosome 9p21 encodes for p16 tumor suppressor protein that has been discussed as a prognostic factor in breast cancer. It inhibits cyclin-dependent kinases (CDKs) 4 and 6 at the G1 to S-phase transition of the cell cycle and thus prevents phosphorylation of the retinoblastoma (RB1) protein (Sherr et al, 2004). Maintaining hypophosphorylation of RB family members promotes binding to E2F1 and leads to G1 cell cycle arrest (Li et al, 2011). Owing to this, p16 is fundamental in various tumor types including colon cancer, melanoma and gallbladder (Romagosa et al, 2011). Moreover, a vital role of p16 expression is suggested in breast cancer for tumor progression (Milde-Langosch et al, 2001; Witkiewicz et al, 2011; Karray-Chouayekh et al, 2011; Hui et al, 2000), metastasis (Hui et al, 2000), and clinical outcome (Karray-Chouayekh et al, 2011; Hui et al, 2000). It is, thus, remarkable that the chromosomal region 9p21, is potentially related to breast cancer

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progression (Oliveira et al, 2010; Haverty et al, 2008; Silva et al, 2003; Brenner and Aldaz, 1995, Schwendel et al, 1998; Loo et al, 2008; Cairns et al, 1995).

Secondly, inactivation of *CDKN2A* gene has been shown to involve four types of genetic alterations: homozygous deletion, promoter hypermethylation, loss of heterozygosity and point mutation, of which homozygous deletion and promoter hypermethylation constitute most of the alterations (Tam et al, 2013). Further analysis has revealed that the gene is frequently homozygously lost or deleted in many tumor types, including glioma (Zadeh et al, 2007), bladder carcinoma (Abat et al, 2014), lung carcinoma (Tam et al, 2013), esophageal carcinoma (Qureshi et al, 2012), renal carcinoma (Ikuerowo et al, 2007), and breast carcinoma (Lebok et al, 2016). Hence, it is ascertained to elucidate the incidence of *CDKN2A* gene deletion and analyze its correlation with clinicopathological parameters of breast cancer patients.

MATERIALS & METHODS

Patients:

In the present study, a total of 30 untreated histologically confirmed breast cancer patients registered at The Gujarat Cancer & Research Institute were enrolled. The study was approved by the Institute's Ethics Committee Board and general consent forms were obtained from all the patients. Detailed clinicopathological history of the patients was obtained. Histopathological details such as tumor size, lymph node status, disease stage, Bloom-Richardson score (BR score),

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histological grade and status of ER, PR, Her2 were evaluated and reported on routine basis by the pathologists of our institute. The clinicopathological characteristics of the enrolled patients are enlisted in Table 1. All breast cancer patients in the present study were of Invasive Ductal Carcinoma (IDC) type.

Fluorescence *in-situ* Hybridization (FISH)

For detection of 9p21 deletions, FISH analysis was carried out on formalin fixed paraffin-embedded tumor tissue blocks of the enrolled breast cancer patients using commercially available ZytoLight FISH Tissue Implementation pretreatment kit. The tumor tissue sections were then deparaffinized, dehydrated with alcohol series (100%, 90%, 70%), incubated in pre-warmed heat pre-treatment solution (PT1) at 90° C. Thereafter drop wise Pepsin Solution (ES1) was applied to the tissue section and incubated for 18 min at 37 °C in a humidified chamber followed by washes in Wash Buffer (WB1). Subsequently, appropriate amount of Locus specific identifier (LSI P16 Spectrum Red/centromeric enumeration probe (CEP) 9 green dual color probe (Cytocell P16 CDKN2A) was applied. The tissue sections were incubated at 75°C for 12 min to carry out denaturation and then at 37°C for overnight hybridization in the humidified chamber. Post-hybridization washes were carried out next day in 1X Wash Buffer and dehydrated with alcohol series, followed by counterstaining with DAPI. The slides scanning and capturing was done using OLYMPUS BX61 fluorescent microscope (OLYMPUS BX61, Japan) at a magnification of 100X. Total 20 randomly selected invasive tumor cells were evaluated for interpretation. The specimens having 2 orange and 2 green signals (2O2G) were considered to have normal intact *CDKN2A* gene. Loss of one orange signal resulting in 1O2G pattern were regarded as homozygous deletion of *CDKN2A*

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while specimens having only 2 green signals (2G) were categorized as hemizygous *CDKN2A* deletion.

Statistical analysis:

The statistical evaluation of the data was carried out using SPSS Inc. version 16 software. Two-tailed chi square test and spearman's correlation method was used to correlate the *CDKN2A* gene expression with various clinicopathological characteristics of breast cancer patients. P≤0.05 was considered to be statistically significant.

RESULTS

Clinicopathological parameters of Breast Cancer patients

As shown in Table 1, breast cancer patients enrolled in the study were in the age group of 21 to 71 years and the median age of patients was 44 years. Median age was used as a cut off to categorize the patients into younger (≤44) and older age group (>44). Accordingly, 17 (57%) patients were in younger age group and 13 (43%) patients were in older age group. Among all 30 patients of breast cancer, 26 (87%) were females and 4 (13%) were males. For lymph node status, in 15 patients (50%) there were no sign of it, while lymph node was involved with tumor cells in 15 patients (50%). BR score was used to divide the patients into groups having grade 1 tumors (well-differentiated), grade 2 tumors (moderately differentiated) and grade 3 tumors (poorly differentiated). Accordingly, 9 patients (40.9%) were having grade 2 tumors

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and 13 patients (59.1%) had grade 3 tumors. BR score data was not available for the rest 8 patients. In addition, vascular permeation was absent in 16 patients (53%), and observed in 14 patients (47%). Perineural invasion was absent in 24 patients (80%) but observed in 6 patients (20%) and perinodal extension was absent in 18 patients (60%), whereas seen in 12 patients (40%). Eleven patients (37%) were ER and PR negative, and 19 patients (3%) were ER and PR positive. Of total patients, none patient was Her2 negative, whereas 28 patients (90.35%) were Her2 equivocal and 2 (6.7%) patients were Her2 positive. Further, 1 patient (3.3%) was in stage I, 6 patients (20%) had stage II and 11 patients (36.6%) were with stage III breast cancer.

Table: 1 - Clinicopathological parameters of Breast Cancer patients

Variables	**No. of patients**	**(%)**
Age Range	**21 - 71 years**	

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Age	≤44	17	57%
(years)	>44	13	43%
Gender	Male	4	13%
	Female	26	87%
Lymph node status	Absent	15	50%
	Present	15	50%
BR Score*	3 to 5 (grade 1)	None	-----
	6 to 7 (grade 2)	9	41%

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	8 to 9 (grade 3)	13	59%
Vascular permeation	Absent	16	53%
	Present	14	47%
PNI status	Absent	24	80%
	Present	6	20%
PNE status	Absent	18	60%
	Present	12	40%
ER	Negative	9	30%

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	Positive	21	70%
PR	Negative	9	30%
	Positive	21	70%
Her2	Negative	0	00
	Equivocal	28	93%
	Positive	2	7%
STAGE	Stage I	1	3%
	Stage II	6	20%

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	Stage III	11	37%
	Unknown	12	40%

*BR score data was not available for 8 breast cancer patients

CDKN2A gene status by FISH:

In total breast cancer patients, *CDKN2A* gene was found to be deleted in 73% (22/30) patients, whereas 27% (8/30) had intact *CDKN2A* gene. Moreover, of the total patients found to have *CDKN2A* gene deletion, 77% (17/30) patients were found to have hemizygous deletion and 23% (5/30) patients had homozygous deletion (Table 2). Representative images are shown in Figure 1.

Table 2: FISH result for *CDKN2A* gene status

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Malignancy	Gene status		
	Normal N (%)	Deleted N (%)	
Breast Cancer	8 (27%)	22 (73%)	Hemi – 17 (57%)
			Homo – 5 (16%)

Correlation of *CDKN2A* FISH results with clinicopathological parameters in Breast Cancer

As depicted in Table 3, correlation of *CDKN2A* gene status with clinicopathological parameters of breast cancer patients was studied. It was revealed that deletion of *CDKN2A* gene was significantly associated with grade 2 (moderately differentiated) breast tumors (p=0.02) as compared to grade 3 tumors (poorly differentiated). Moreover, incidence of *CDKN2A* gene deletion was significantly higher in patients without vascular permeation (p=0.04), perineural invasion (p=0.01) and perinodal extension (p=0.03) as compared to those having vascular permeation, perineural invasion

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and perinodal extension, respectively. However, no other significant associations were observed between *CDKN2A* gene deletion and rest of the studied parameters.

Table: 3 Correlation of *CDKN2A* gene with clinicopathological parameters in Breast Cancer

Variables		***CDKN2A* gene status**				
	N (%)	**Normal** **N (%)**	**Deleted** **N (%)**	**χ2**	**r**	**P**
	30 (100)	8 (27)	22 (73)			
Age (years)						
≤ 44years	17 (57)	3 (18)	14 (82)	2.3	-0.27	0.13

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> 44 years	13 (43)	5 (39)	8 (61)			
Gender						
Male	4 (13)	1(25)	3(75)	1.04	0.09	0.6
Female	26 (86)	7 (26)	19 (64)			
Lymph Node Status						
Absent	15 (50)	3 (21)	10 (79)	2.35	-0.25	0.17
Present	15 (50)	5 (33)	10 (67)			
Stage						
Stage I	1 (3)	0 (00)	1 (100)			

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Stage II	5 (17)	2 (40)	3 (60)	10.7	0.33	0.16
Stage III	12 (40)	3 (25)	9 (75)			
BR Score						
6 to 7 (Grade 2)	9 (41)	1 (11)	8(89)	5.8	-.47	**0.02**
8 to 9 (Grade 3)	13 (59)	5 (38)	8 (62)			
Vascular Permeation						
Absent	16 (53)	2 (12)	14 (88)	4.2	-3.7	**0.04**
Present	14 (47)	6 (43)	8 (58)			
Perineural Invasion						

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Absent	24 (80)	4 (17)	20 (83)	6.47	-.43	**0.01**
Present	6 (20)	4 (66)	2 (33)			
Perinodal Extension						
Absent	18 (60)	2 (12)	16 (88)	5.7	-.39	**0.03**
Present	12 (40)	6 (50)	6 (50)			
ER Status						
Negative	9 (30)	4 (45)	5 (55)	1.6	-.205	0.29
Positive	21 (70)	4 (19)	17 (81)			
PR Status						

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Negative	9 (30)	4 (45)	5 (55)	3.07	-.08	0.6
Positive	21 (70)	4 (19)	17 (81)			
Her2 status						
Negative	-------	--------	---------	1.6	0.04	0.8
Equivocal	28 (93)	8 (29)	20 (71)			
Positive	2 (7)	00(0)	2 (11)			

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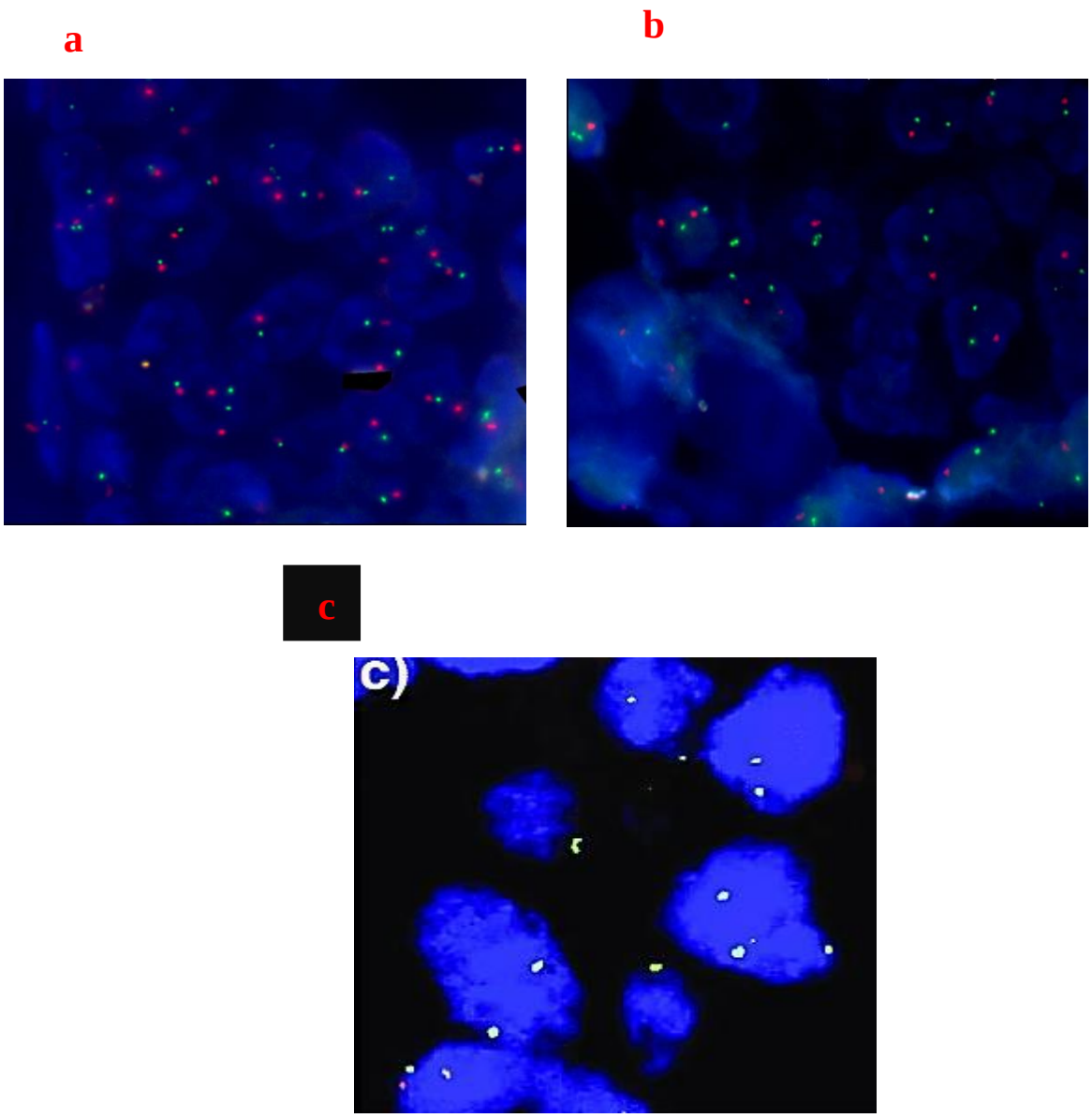

Figure-1: Representative images for signal patterns observed in FISH assay

(a) Normal pattern of *CDKN2A* gene (b) Hemizygous deletion of *CDKN2A* gene (c) Homozygous deletion of *CDKN2A* gene

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DISCUSSION

CDKN2A is established as a tumor suppressor gene that encodes a specific inhibitor of cyclin-dependent kinase (CDK) 4 and 6, critical for cell cycle regulation. It plays a pivotal role in tumor suppressor networks through inducing cellular senescence that acts as a barrier to cellular transformation by oncogenic signals (Kotake et al. 2015). P16 protein encoded by *CDKN2A* has been implicated in processes such as apoptosis, cell invasion and angiogenesis. However, *CDKN2A* gene is found altered in a wide range of human cancers with loss of p16 in many malignant disorders, although overexpression of p16 is demonstrated in some tumors (Romagosa et al, 2011). Additionally, the loss of p16 may be an early event in cancer progression, because deletion of at least one copy is quite high in some premalignant lesions (Liggett et al, 1998). Allelic losses of 9p21 are detected and conflicting data exist whether these changes develop early or late in the malignant progression (Gonzalez et al, 1997). Therefore, the present study aimed to detect the *CDKN2A* gene deletion in breast cancer patients.

A total of 30 breast cancer patients who underwent surgery at The Gujarat Cancer and Research Institute were included. *CDKN2A* gene was found to be deleted in 73% (22/30) and was intact in 27% (8/30) of breast cancer patients with hemizygous deletion found in 57% (17/30) and homozygous deletion in 16% (5/30) of total patients. In a study by Aftab et al (2018) entire gene deletions were observed in approximately 42.8% of breast cancers. There

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are still other earlier studies which have reported 9p deletions in 6–25% breast cancers by array CGH (Haverty et al, 2008; Jong et al, 2004; Seute et al, 2001). Moreover, higher rates of 41% of 9p deletions were found in metastatic breast cancers or in studies employing less quantitative loss of heterozygosity (de Oliveira et al, 2010; 13, Silva et al, 2003; Brenner & Aldaz, 1995; Cairns et al, 1995. However, Lebok et al (2016) analysed 2,197 breast cancers and found 9p21 deletions in 15.3% interpretable breast tumors by FISH. The deletions observed by Lebok et al (2016) were 13.6% heterozygous and 1.7% homozygous deletions.

In addition, *CDKN2A* gene deletion has been reported in other malignancies too. Jacobsen et al (2020) observed the gene deletion in 32.1% adenocarcinoma (EAC) and 33.5% squamous cell carcinoma (SSC) esophageal cancer patients by IHC and FISH. Also, Kotzev and Kamenova (2017) detected loss of *CDKN2A* gene in 69% esophageal adenocarcinoma specimens by FISH, including hemizygous deletion occurring with highest frequency in 54% EAC specimens whereas homozygous deletion presented in 15% EAC specimens. This is similar to the incidence of *CDKN2A* gene deletion observed in the present study. Fahmy et al (2004) too has found noticeable loss of the 9p21 signals in 90% of analyzed EAC samples by FISH. Also, in gastric cancer, *CDKN2A* deletions were found in 38.9% of the tested specimens (Qiao et al, 2021), while in 59% of non-small cell lung tumors making it the most frequent mechanism of inactivation (Tam et al, 2013).

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Next, the correlation of *CDKN2A* gene status with the clinicopathological parameters of breast cancer patients revealed significant inverse association of *CDKN2A* gene deletion with tumor grade, vascular permeation, perineural invasion and perinodal extension. Contradictorily, Lebok et al observed that 9p21 deletion was linked to adverse tumor features, including high-grade ($p < 0.0001$) and nodal positive cancers ($p = 0.0063$), high cell proliferation ($p < 0.0001$), negative hormone receptor (ER/PR) status ($p \leq 0.0006$), and HER2 amplification ($p = 0.0078$) in breast cancer patients (Lebok et al, 2016). On the other side, Jacobsen et al (2020) did not find any correlation of *CDKN2A* gene deletion with the clinicopathological parameters of esophageal cancer patients, but the study revealed correlation of p16 protein expression with low tumor stage and better overall survival. Further, Malerova et.al. (2020) studied the significance of p16 protein expression in relation to clinicopathological parameters and prognosis in patients with oral squamous cell carcinomas and observed that P16 positivity seems to be a negative prognostic factor in oral carcinomas. This is in line with the results obtained in the present study, showing that the CDKN2A gene deletion is associated with good prognostic factors.

A probable reason for the conflicting results of *CDKN2A* gene deletion could be that there are variants of the gene present due to single nucleotide polymorphism which predisposes to malignant transformation (Dębniak et al, 2005). Thus presence of different variants might lead to distinct associations with the clinicopathological parameters. The present study has observed significant associations of *CDKN2A* gene deletion with good prognosticators, indicating that the gene

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deletion could be an early event in carcinogenesis. This is supported by the previous studies showing that alterations of p16 occur early during tumorigenesis as they are commonly seen in Barret's dysplasia and peritumoral mucosa (Jacobsen et al, 2020). Moreover, Dębniak et al (2005) has suggested *CDKN2A* to be a low penetrance breast cancer susceptibility gene in Poland (Dębniak et al, 2005). Hence, on the basis of previous studies and present study result, P16 overexpression - but not expression loss was linked to adverse tumor parameters (Lebok et al, 2016). Moreover, FISH technique is considered the gold standard for gene copy number analysis. This is because it allows for precise gene copy number determination in individual cells rendering it independent of cancer tissue purity or aneuploidy.

CONCLUSION:

Although *CDKN2A* gene is well-known for its role as a tumor suppressor gene, the present study observed that the gene deletion was associated with absence of known worse prognosticators indicating that the gene expression and not loss was linked to adverse tumor parameters. Moreover, since *CDKN2A* gene deletion is considered as an early event in carcinogenesis, the association with good prognostic factors suggests the same. Hence, detection of *CDKN2A* gene deletion could help to stratify the specific subsets of patients into high risk and low risk groups. Secondly, FISH technique allows for precise gene copy number determination in individual cells, rendering it independent from the purity of cancer tissues or presence of aneusomy. FISH is thus considered the gold standard for gene copy number analysis.

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THE GUJARAT CANCER & RESEARCH INSTITUTE
(M. P. SHAH CANCER HOSPITAL) AHMEDABAD-380 016.
ધી ગુજરાત કેન્સર એન્ડ રિસર્ચ ઈન્સ્ટિટ્યૂટ
(એમ.પી.શાહ કેન્સર હોસ્પિટલ) ન્યૂ સિવિલ હોસ્પિટલ કંપાઉન્ડ, અસારવા, અમદાવાદ-૩૮૦ ૦૧૬.

Regd. No.
Name :
Age/Sex :

STICKER

અમો નીચે સહી કરનાર (દર્દી)_____

અને દર્દીના સગા _____ જણાવીએ છીએ કે અમોને ડૉકટરશ્રીએ બિમારી વિશે તથા તેના પરિણામોની સંપૂર્ણ માહિતી આપેલ છે. તે સંબંધે નીચે જણાવેલ તપાસ સારવારમાંથી જે કોઈપણ જરૂરી હોય તે કરવાની અમો ડૉકટરને પરવાનગી આપીએ છીએ.

૧. નિદાન માટે જરૂરી બાયોપ્સી, એન્ડોસ્કોપી, એન્જીયોગ્રાફી, સીટી સ્કેન, સોનોગ્રાફી વગેરે માટે.
૨. કોઈપણ પ્રકારના ઓપરેશન અને તેના માટે જરૂરી એનેસ્થેશીયા માટે.
૩. કોઈપણ પ્રકારની રેડીયોથેરાપી સારવાર માટે.
૪. કેમોથેરાપી સારવાર માટે.
૫. સારવાર દરમ્યાન લોહી અથવા લોહીના કમ્પોનેન્ટસ આપતી વખતે સંપુર્ણ તકેદારી રાખવા છતા કોઇ અનિચ્છનિય પરિણામ આવે તોતે પણ અમો સ્વીકારવા તૈયાર છીએ.
૬. અમો લોહીના નમુના, ટીસ્યુના નમુના તથા તપાસ કે જે ગુ.કે.રી.ઇ અથવા ગુ.કે.સો. દ્વારા ચાલતા સંશોધન કાર્ય માટે પણ આપવા સંમત છીએ.

દર્દીની સહી _____ દર્દીના સગાની સહી _____

તારીખ _____ તારીખ : _____

हम (मरीज) _____

और (मरीज के रीस्तेदार) _____

स्वीकार करते है की बिमारी की चिकित्सा एवं उससे होने वाले संभावित परिणाम के विषय में हमें पुरी जानकारी दी गई है ईस सम्बन्ध हम निम्न प्रक्रियाओ के लिए अनुमति देते है।

१. निदान के लिये आवश्यक प्रकिया जैसे कि बायोप्सी, एन्डोस्कोपी, एन्जियोग्राफी, सीटी स्केन ईत्यादी
२. किसी प्रकार की शस्त्रकिया एवं बेहोश करने की आवशयकता के लिये.
३. विकिरण चिकित्सा (रेडियोथेरापी) : ब्राह्य एवं आन्तरिक (ब्रेकी थेरापी).
४. औषधो चार (कीमोथेरापी) की दवाओ के लिये.
५. संस्था एवं केन्सर सोसायटी के रीसर्च के लिये, रक्तपेशी, ईत्यादी के सेम्पल या कोई भी जांच के लिये
६. मुझे ज्ञान है की चिकित्सा के समय, मुझे रक्त अथवा रक्तपेशी की आवश्यका हो सकती है, हालाकी ईसमें सभी प्रकार की सावधानी ली जाती है, फिर भी कुछ दुश्यपरिणाम हो सकते है जो हमें स्वीकार्य है.

मरीज के हस्ताक्षर _____ मरीज के रीस्तेदार के हस्ताक्षर _____

दिनांक : _____ दिनांक : _____

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